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Previously we demonstrated expression of vasopressin and oxytocin gene-related products in breast cancer, but not benign fibrocystic breast disease, using the technique of immunohistochemistry. In vitro and in vivo studies suggest that both of these hormones have growth regulatory effects on breast cancer cells. Therefore, these hormones may represent autocrine/paracrine and/or endocrine factors for breast cancer. However, the receptors through which these hormones act and the effector mechanisms involved with cancer cell growth are not known. Using RT-PCR we demonstrated the expression of mRNA for the 4 known vasopressin receptors, including the novel VACM receptor, and the oxytocin receptor. During this last year we cloned VACM from cancer cells (NCI-H146 and MCF7), using the technique of 5' and 3'-RACE. The isolated clones contains an open reading frame of 2,343 nucleotides and encode a protein of 781 amino acids. Obtaining the cDNA for VACM will aid in determining the role of this receptor in breast cancer pathophysiology. In addition we have demonstrated vasopressin-induced calcium mobilization in breast cancer cells, and preliminary results indicate vasopressin-induced activation of MAPK. The studies suggest that vasopressin may stimulate cancer cell growth through VACM, V_{1a}, or V_{1b} vasopressin receptors by activating the MAP kinase cascade.

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Michael J. Fay 9/25/97

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(4) TABLE OF CONTENTS

(5) INTRODUCTION

Vasopressin and oxytocin are nine amino acid hormones (nonapeptides) which are produced by distinct neurons in the paraventricular and supraoptic nuclei in the hypothalamus of the brain. The major physiological roles for vasopressin (antidiuretic hormone) include antidiuresis, vasopressor effects, and modulation of adrenocorticotropic hormone (ACTH) release from the anterior pituitary. The physiological roles for oxytocin include contraction of smooth muscle cells which result in lactation, and the uterine contractions which are important for parturition. These diverse actions of vasopressin and oxytocin are mediated by a number of specific cell-surface receptors which will be discussed below. Like many other peptide hormones, vasopressin and oxytocin are produced as prohormones, which are packaged into neurosecretory vesicles. In these vesicles the prohormones are acted on by processing enzymes which result in the generation of distinct peptide products. Processing of the vasopressin precursor results in the generation of three distinct gene-products (vasopressin, vasopressin-associated neurophysin, and vasopressin-associated glycopeptide) (North, 1987) Processing of the oxytocin precursor results in the generation of the active hormone and an oxytocin-associated neurophysin (North, 1987). These neurosecretory vesicles, containing the vasopressin and oxytocin gene-related products, come to reside in the axonal terminals of neurons which are located in the posterior pituitary. Physiological stimulation causes an action potential in these neurons which results in the release of active hormone and distinct gene- products into the circulation. In addition to the production of these hormones within the CNS, a number of studies suggest that peptide hormones, like oxytocin and vasopressin, can be produced by a number of peripheral tissues. It is thought that this peripheral production of oxytocin and vasopressin may represent an autocrine/paracrine role for these peptides in addition to their well established endocrine roles (Wathes and Swann, 1982; Ivell and Richter, 1984; Nicholson et al., 1984; Nussey et al., 1984; Wathes, 1984; Ivell et al., 1985; Schwams et al., 1985).

It has been known for some time that certain cancers, such as small-cell lung carcinoma, can ectopically produce neuropeptide hormones like oxytocin and vasopressin (Sausville et al., 1985; North, 1991; North et al., 1995). Research performed in this laboratory, using the technique of immunohistochemistry and archival breast cancer and fibrocystic breast disease specimens, indicates that expression of vasopressin and oxytocin gene-related products is a common feature of breast cancer (North et al., 1995), but not benign fibrocystic breast disease (Fay et al., submitted 1997). The ability of breast cancer cells to process peptide prohormones and generate active hormone, like vasopressin and oxytocin, is supported by the recent finding that both breast cancer cell lines and biopsy specimens express prohormone processing enzymes (Cheng et al., 1997). In many cases this ectopic production and secretion of hormone can result in paraneoplastic syndromes which are characterized by clinical symptoms resulting from hormone excess (Sorenson et al., 1995). In further support of a connection between vasopressin and breast cancer, there are two published clinical case reports of breast cancer patients presenting with the syndrome of innapropriate antidiuretic hormone secretion (SIADH) (Gupta et al., 1986; Howard et al., 1993). Collectively, these findings suggest that neuropeptide expression/secretion by breast cancer cells may represent a potential diagnostic biomarker

for this cancer, and suggests a possible role of neuropeptids as cancer cell autocrine/paracrine factors. In addition, the expression of unprocessed or modified neuropeptide gene-products on the cell surface of the cancer cells might prove to be a potential therapeutic target as in small-cell lung cancer (North et al., 1989; Rosenbaum et al., 1990; North and Yu, 1993; see initial progress report for cell surface vasopressin-like immunoreactivity in MCF7 breast cancer cells).

The diverse physiological actions of vasopressin and oxytocin are carried out by the binding of hormone to cell surface receptors located in specific target tissues. In the case of vasopressin, 4 cell surface receptors have been identified and cloned. The antidiuretic actions of vasopressin occur through activation of renal V_2 type receptors (Birnbaumer et al., 1992). The V_2 type vasopressin receptor belongs to the 7-transmembrane superfamily of G-protein coupled receptors, and activation results in an increase in adenylate cyclase activity, increases in cAMP, and activation of protein kinase A. The cloned V_{1a} and V_{1b}/V_3 vasopressin receptors are also 7-transmembrane G-protein coupled receptors (Keyzer et al., 1994; Hirasawa et al., 1994; Thibonnier et al., 1994). The V_{1a} vasopressin receptor is located in vascular smooth muscle and is responsible for the vasopressor effects of vasopressin, while the V_{1b}/V_3 vasopressin receptor is located in the anterior pituitary and is responsible for modulating the release of adrenocorticotropic hormone (ACTH). Both receptor types couple to phospholipases, and receptor activation results in the generation of inositol phosphates, rises in intracellular calcium, and activation of protein kinase C. In addition to these vasopressin receptors, a novel vasopressin receptor was recently cloned, and was named the vasopressin-activated calcium mobilizing receptor or VACM (Burnatowska-Hledin et al., 1995). This receptor is unique in that hydrophobicity plots indicate that it does not belong to the 7-transmembrane G-protein coupled receptor superfamily. In addition, upon vasopressin binding/interaction this receptor increases intracellular calcium by an as yet undefined mechanism. The cloned human oxytocin receptor is also a member of the 7-transmembrane G-protein coupled receptor superfamily, and in the physiological setting activates signal transduction pathways similar to the V_1 vasopressin receptors (Kimura et al., 1992).

For neuropeptides like vasopressin and oxytocin to serve as autocrine/paracrine and/or endocrine growth regulatory factors for breast cancer cells, the expression of vasopressin receptor subtypes and functionality of these receptors must be determined. As presented in last years progress report and in the enclosed abstract for the DODErа of Hope meeting, I have demonstrated the expression or mRNAs for vasopressin receptors and for the oxytocin receptor in a number of cultured breast cancer cell lines. In addition, I have also demonstrated vasopressin-induced calcium mobilization in cultured breast cancer cell lines. Collectively these results suggest that vasopressin, acting through V1 or the recently identified VACM receptor, influences breast cancer cells by raising intracellular-free calcium. Such findings are consistent with the research of Taylor et al (1990), who found that vasopressin increased levels of total inositol phosphates in cultured breast cancer cells. These findings indicate that breast cancer cells express a number of neuropeptide receptor subtypes, and indicate vasopressin receptor functionality. I was unable to demonstrate oxytocin-induced calcium mobilization, despite the expression of mRNA for this receptor in the cell lines examined. Recently Cassoni et al.

(1997), demonstrated that oxytocin induced the accumulation of cAMP, but not calcium mobilization, in the MDA-MB-231 breast cancer cell line. This rise in cAMP was also correlated with a growth-inhibitory effect of oxytocin (Cassoni et al., 1994), these finding are in contrast to the growth promoting influences found by Taylor et al. (1990) for oxytocin, which were correlated with increases in total inositol phosphates. The findings of Cassoni et al. (1997) corroborates the lack of oxytocin-induced calcium mobilization we observed in other breast cancer cell lines (Zr-75 and T47D), and implicates that oxytocin receptor coupling and signal transduction in breast cancer cells is regulated differently than in normal oxytocin target tissue. One possible explanation for the discrepencies in oxytocin receptor signal transduction in breast cancer cells may be coupling of the receptor to multiple G-proteins (Milligan, 1993), or cross talk between signal transduction pathways. I am in the process of preparing the oxytocin receptor RT-PCR data and oxytocin-induced lack of calcium mobilization data for publication.

Vasopressin has been shown to stimulate breast cancer cell growth both in vitro (Taylor et al., 1990), and in vivo (Chooi et al., 1994). However, the receptors and signal transduction mechanisms have not been elucidated. The RT-PCR results we have obtained suggest that vasopressin may be acting at several receptors to modulate cancer cell growth, and not just the V1a receptor as has been postulated by others (Chooi et al., 1994). The sequence of signal transduction events associated with vasopressin-induced breast cancer-growth are also unknown. Taylor et al (1990) correlated a rise in intracellular inositol phosphates with vasopressin-induced growth of breast cancer cells, and we have demonstrated increases in intracellular free calcium. These signal transduction events suggest activation of vasopressin V_1 receptors and/or activation of VACM receptors. In addition to the growth inhibition found with oxytocin-receptor activation (Cassoni et al., 1994), the expression of V2 receptors by cancer cells may represent a possible negative growth regulatory mechanism, since cAMP has been shown to inhibit the growth of breast cancer (Tagliaferri et al., 1988). One possible mechanism by which neuropeptides may influence cancer cell growth is by activating the mitogen-activated protein kinase (MAPK) cascade, a pathway common to growth promoting stimuli (Davis 1993; L'Allemain, 1994 ; Cobb and Goldsmith, 1995). In Physiological settings both oxytocin and vasopressin are capable of activating the MAP kinase cascade (Kribben et al., 1993; Ohmichi et al., 1995; Nohara et al., 1996), and preliminary findings by this laboratory support the idea that vasopressin may be stimulating MAPK phosphorylation in cultured breast cancer cells.

(6) BODY

A. Immunohistochemical detection of vasopressin gene-related products in benign fibrocystic disease

We have submitted our initial results on the immunohistochemical evaluation of vasopressin gene expression in benign fibrocystic disease to Endocrine Pathology for consideration for publication (see last years progress report for preprint of paper). This paper was deemed acceptable for publication pending revisions and some additional research. The reviewers for this journal wanted to see cases of acetone-fixed fibrocystic

disease stained for vasopressin gene-related products, in addition to the formaledyde-fixed tissues we examined, since the original breast cancer work we published was performed using acetone-fixed breast cancer specimens (North et al., 1995). In addition, the reviewers wanted to see staining for vasopressin gene-related products performed on cancer biopsy specimens from patients who were initially diagnosed with benign fibrocystic disease. We have initiated the process of locating the archival tissue specimens, preparing slides from the blocks, and staining the tissue sections using our antibody to vasopressin-associated glycopeptide. We are in the process of performing dilution trials with the anti-vasopressin associated glycopeptide antibody, and confirming tissue pathology and staining with Dr. Vincent Memoli of Dartmouth Hitchcock Medical Center. When these immunohistochemical experiments are finished the paper will be revised and resubmitted to Endocrine Pathology. The continuing work on the immunohistochemical detection of vasopressin-gene related products in breast cancer and benign fibrocystic bresat disease addresses technical objective 1 of the original grant application.

B. Cloning of a novel calcium mobilizing vasopressin receptor from cancer cells (NCI-H146 small cell-lung cancer cells and MCF7 breast cancer cells).

As mentioned in the last progress report I was able to demonstrate expression of mRNA for the novel vasopressin receptor, called VACM, in breast cancer cell lines using the technique of RT-PCR and primers designed against the rabbit VACM sequence. Since that time a human clone of VACM was published by a British research team (Byrd et al., 1997; Stankovic et al., 1997). In order to study the role of this receptor in cancer cells, a large laboratory effort was undertaken to obtain a cDNA clone for this receptor from human cancer cells. These studies address technical objective 3 of the original grant application. Initial efforts were focused on the small-cell lung carcinoma cell line NCI-H146, since we had obtained signal transduction results supporting the expression of functional VACM in this cell line. However, we have subsequently obtained the 5' and 3' RACE (rapid amplification of cDNA ends) products, covering the entire open reading frame of this gene, from the MCF-7 breast cancer cell line. The procedures used to isolate a cDNA clone of VACM from human cancer cells are as follows. Approximately 1 μg of total RNA was subjected to reverse transcription using the superscript II enzyme (Life Technologies), and the 138 primer (see below). The RACE PCR technique was performed using the primers listed below and Pfu polymerase (Stratagene). The conditions used were as follows: an initial denaturation step at 96 °C for 2.5 min; 35 cycles of 96 °C for 45 sec, 55 °C for 45 sec, and 72 °C for 8 min; followed by a final extension of 20 min. The generated products (~2.1 kb and 1.6 kb) were cloned into the pZERO-(Blunt) vector (Invitrogen). Sequencing of the generated clones was performed using an ABI/Prism automated sequencing system (Perkin Elmer). Sequence analysis was performed using Align/Megalign (DNAStar, Inc.), and Gene Inspector (Textco, Inc.) software programs, and protein motif analysis was performed using the Internet version of MotifFinder (Institute for Chemical Research, Kyoto University).

3'-PCR RACE primes for VACM:

1432(forward): 5' gaa-tgg-cta-aga-gaa-gtt-ggt-atg 3'
138(reverse): 5' ttg-ttt-ttg-taa-ggt-aag-gca-gag 3'

5'-PCR RACE primers for VACM:

5'ATG (forward): 5' tcc-aag-tta-aag-aac-atg-gcg 3'
2082 (reverse): 5' tct-tct-ctc-atc-ctt-tct-gta-gtg 3'

The isolated clone for NCI-H146 contains an open reading frame of 2,343 nucleotides and encodes a protein of a predicted size of 781 amino acids. Analysis programs failed to identify hydrophobocity regions of significance to classify them as transmembrane regions. Motifs identified included two protein kinase A phosphorylation domains (Thr 427 and Ser 731), 15 protein kinase C phosphorylation sites, a tyrosine phosphorylation site Tyr 207, two myristoylation sites contained by residues 180..185 and 664..669, and three N-glycosylation sites at Asn 145, Asn 289, and Asn 566. These findings are unpublished, and we are in the process of preparing the manuscript. The cDNA sequence for HVACM from human cancer cells has been submitted to the Genebank by us and has an assigned acession number of AF017061. A complete copy of the gene bank submission is included in the appendices of this progress report. Now that we have cloned VACM from a human cancer cell line, we have the molecular information needed to examine in detail the expression of this mRNA and protein, so that it's interactions with vasopressin and role in breast cancer cell biology can be fully examined.

C. Vasopressin-induced phosphorylation (activation) of mitogen-activated protein kinase (MAPK).

In an effort to further examine the influence of vasopressin on activation of MAPK in MCF7 breast cancer cells I have devoted a considerable amount of time to the development of a fluorescent western-blot protocol which we have attempted to quantify using a recently purchased Molecular Dynamics Fluorimager. These studies address technical objective 3 of the original grant application. The materials and methods used for these experiments are as follows. MCF7 breast adenocarcinoma cells were obtained from the American Type Culture Collection (ATCC HTB22). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS; Hyclone) at 37 °C in an environment of 5% CO_2. Every 2-3 days the cells either received fresh growth medium or were subcultured using 0.25% trypsin/0.02% EDTA. For experimental purposes, cells were trypsinized, counted using trypan blue and a hemocytometer, and 2 x 10^6 viable cells were seeded into 6-well plates (Costar) in 3 ml of DMEM containing 1% FBS. The cells were plated out in these reduced serum conditions to decrease MAPK activity (phosphorylation). These conditions did not appear

to influence cell attachment to the plates. On the day of experimentation, the media was aspirated and replaced with DMEM containing 0.5% FBS for 2 hours. This was done to eliminate possible endogenous autocrine/paracrine growth factors secreted by the cancer cells. The cell were then treated for 5 or 15 minutes with 1 μM arginine vasopressin (AVP; Calbiochem). The working concentration of AVP was obtained by a 1:1,000 dilution of a 1 mM stock in 0.05 M glacial acetic acid. Controls for these experiments included a DMEM/0.05% FBS media control, and an acetic acid control (DMEM/0.05% FBS + 50μM glacial acetic acid). After AVP treatment (5 or 15 min) at 37 °C, experimental solutions were aspirated, and cells lysed in 100 μl of boiling SDS sample buffer containing 62.5 mM Tris HCl (pH 6.8), 2% w/v SDS, 10% glycerol, 50 mM DTT, and 0.1% w/v bromphenol blue. The lysed cells were scraped into 1.5 ml eppendorf tubes, boiled for 5 min, and passed through a 26 gauge needle to reduce viscosity. A 20 μl aliquot of each sample was electrophoresed at 75 volts for 3.5 hours using a 12% polyacrylamide separating gel. Controls samples consisting of 5 μl of either non-phosphorylated p42 MAPK or dually phosphorylated p42 MAPK (New England Biolabs) were also evaluated. A prestained protein ladder (10 μl) was also run on each gel as a means of determining relative protein mass (BioRAD). After electophoresis, proteins were transferred to a PVDF-plus membrane (MSI) at 15 volts for 12 hours at 4 °C.

The membranes were then subjected to Western blotting using antibodies which recognize dually phosphorylated MAPK (activated MAPK), and MAPK regardless of phosphorylation status. Membranes were first blocked at room temperature for 3 hours using 3.5% fish skin gelatin (Sigma) in 10 ml of tris-buffered saline with 2% heat inactivated casein (Sigma). After blocking the membranes were exposed to primary antibody in 10 ml of TBS-tween overnight at 4 °C. The rabbit polyclonal anti-active MAPK antibody recognizes dually phosphorylated p42/p44 MAPK, and was used at a dilution of 1:5,000 (Promega). The rabbit polyclonal antibody which recognizes p42/p44 MAPK regardless of phosphorylation status was used at a concentration of 1:1,000 (New England Biolabs). The next day, primary antibody was decanted, membranes washed 3 x 5 minutes with TBS-tween, and exposed to alkaline-phosphate conjugated goat anti-rabbit (1:2,000) for 1 hour at room temperature. The membranes were then washed 3 x 5 min in TBS-tween, and exposed to 2 ml of ECL substrate (Amersham). The membranes were then wrapped in a non-fluorescent plastic folder, and scanned for fluorescence using a Molecular Dynamics fluorimager, with the photomultiplier tube set at a voltage of 550 for visualization. The data were analyzed using ImageQuant software with corrections made for local background. A ratio was obtained for each sample by dividing the volume of the band reacted with the anti-active MAPK by the volume of the band with the anti-total MAPK. Theoretically this manipulation should equalize the samples for protein loading, and an increase in this ratio demonstrates an increase in MAPK activation (phophorylation). For unknown reasons reliable values were obtained for only the p44 MAPK isoform, and not p42 MAPK, possible explanations include difficulty in determining volumes due to sample distortion (e.g. gel frowning), discrepancies in background adjustments, and close proximity of the p44 and p42 bands. From visual examination of the blots it appeared that there was an increase in activated MAPK with the 5 min vasopressin treatment. Analysis of the data using Imagequant (see Table 1 below) demonstrated a trend of increased ratio for the samples treated with vasopressin

for 5 min. However, more work is needed to verify this method for measuring MAPK activation.

TABLE 1: Ratios of the Band Volumes of Dually Phosphorylated (Active) MAPK to Total MAPK (activated + non-activated) for MCF 7 breast cancer cells treated with 1 μM arginine vasopressin for 5 and 15 minutes.

Sample	P44 MAPK Ratio (5 min)	P44 MAPK ratio (15 min)
Media Control 1	1.72	0.81
Media Control 2	1.70	2.23
Acetic Acid Control 1	2.30	1.93
Acetic Acid Control 2	2.67	2.42
Vasopressin treated 1 (1 μM)	2.72	1.67
Vasopressin treated 2 (1 μM)	8.25	1.64

D. RT-PCR for vasopressin V1a receptor expression in T47D breast cancer cells

As discussed in last years progress report we have initiated an RT-PCR approach to examine expression of vasopressin/oxytocin receptor subtypes in cultured breast cancer cells. Using this approach we were able to demonstrate expression of mRNA for the oxytocin receptor, the V_2 vasopressin receptor, the V_{1b} vasopressin receptor, and the VACM receptor. Initial attempts at amplifying V_{1a} receptor mRNA failed to demonstrate expression of this receptor. However, since that time we have amplified a product of the predicted size of 408 bp from the T47D breast cancer line. This product has not been confirmed by DNA sequencing. The materials and methods used for RT-PCR analyses are as described in last years progress report. These studies address technical objective 3 of the original grant application.

(7) CONCLUSIONS

Previously using the technique of immunohistochemistry and antibodies directed against vasopressin and oxytocin gene-related products we demonstrated that these neuropeptide gene products are commonly expressed in breast cancer (North et al., 1995), but not in benign fibrocystic breast disease (Fay et al., 1997 submitted). These results confirm two published cases of breast cancer patients presenting with the syndrome of inappropriate antidiuretic hormone secretion (Gupta et al., 1986; Howard et al., 1993). These findings suggest that, as in small-cell lung carcinoma, the expression of neuropeptide-related gene- products by cancer cells may be potentially useful diagnostic/therapeutic markers for breast cancer (North et al., 1989; Rosenbaum et al., 1990; North, 1991). This possibility is documented by the fact that using antibodies directed against vasopressin-associated glycopeptide we detect cell surface immunoreactivy in cultured breast cancer cells (see original progress report).

One possible role for the production of small peptide hormones, like vasopressin and oxytocin, by breast cancer cells is that these hormones serve as autocrine/paracrine growth regulatory factors for the cancer cells. This hypothesis seems to be valid since in

vitro (Taylor et al., 1990) and in vivo (Chooi et al., 1994) data indicate that vasopressin stimulates breast cancer growth, and in vitro (Cassoni et al., 1994, 1997) and in vivo (Cassoni et al., 1996) data indicate that oxytocin inhibits breast cancer cell growth. However, the neuropeptide receptors, and the cellular signaling events associated with these hormone-induced growth regulatory effects have not been thoroughly investigated. Work performed in this laboratory (RT-PCR data last progress report) and work published by others (Taylor et al., 1990; Cassoni et al., 1994; Bussolatie et al., 1996; Ito et al, 1996) demonstrate that breast cancer cells express oxytocin receptors. It is of interest that oxytocin receptor activation and growth inhibition of breast cancer cells involves an elevation of cellular cAMP (Cassoni et al., 1997). These results are consistent with our findings of a lack of oxytocin-induced calcium mobilization in the ZR-75 and T47D breast cancer cells. Further research is needed to elucidate the signaling mechanisms involved in oxytocin-induced growth inhibition of breast cancer cells. Such research is of importance since elucidating the mechanisms by which oxytocin causes growth inhibition of breast cancer cells has implications for potential novel therapeutic strategies.

Since vasopressin has been shown to modulate breast cancer cell growth, identifying the receptors involved and the evoked signal transduction pathways is important to understanding how this peptide acts. Research using a cell line derived from a dimethylbenzan(a)anthracene-induced rat mammary tumor demonstrated the presence of V_{1a} vasopressin receptors (Monaco et al., 1978; Monaco et al., 1980; Guillon et al., 1986; Woods and Monaco, 1988). In addition, Chooi et al. (1994) assumed that the growth promotion of vasopressin they found in an in vivo breast cancer model was due to activation of V_{1a} receptors. However, RT-PCR data performed in this laboratory indicates that cultured breast cancer cells appear capable of expressing all 4 cloned vasopressin receptors (VACM, V_{1a}, V_{1b}, and V_2). Therefore the growth promoting influences of vasopressin in breast cancer cells must be reevaluated. The vasopressin-induced calcium mobilization we found in Zr-75 and T47D breast cancer cells could be due to activation of VACM, V_{1a}, or V_{1b} vasopressin receptors, since all these receptors have the potential of increasing levels of intracellular-free calcium. With the cloning of the VACM receptor from cancer cells described in this progress report, we are now able to evaluate the role of the receptor in cancer cell growth by creating breast cancer cell lines which overexpress this receptor. It is of interest that we also find expression of vasopressin V_2 receptor mRNA in breast cancer cells. This receptor could potentially mediate a growth inhibitory effect on breast cancer cell growth since receptor-activation could theoretically raise cellular cAMP levels. and cAMP is growth inhibitory to breast cancer (Tagliaferri et al., 1988). The presence of V2 receptors could potentially explain the paradoxical growth inhibitory influence of vasopressin that Taylor et al. (1990) found for high doses of vasopressin.

In addition to not knowing the receptors involved in vasopressin-induced growth of breast cancer, the signaling mechanisms are also unknown. One potential signaling pathway for vasopressin to influence cancer cell growth is the mitogen activated protein kinase cascade, since G-protein coupled receptors have been shown to stimulate this pathway through a number of signaling mediators (Robbins et al., 1992; Alblas et al., 1993; Howe and Marshall, 1993; Wintz et al., 1993; Seufferlein et al., 1995). Also, both vasopressin and oxytocin have been shown to activate the MAPK cascade in the

physiological setting (Kribben et al., 1993; Ohmichi et al., 1995; Nohara et al., 1996). Preliminary results obtained in this laboratory indicate that vasopressin-induced stimulation of breast cancer cell growth may be due to activation of the MAPK cascade.

This research has demonstrated the expression of neuropeptide related gene-products for vasopressin and oxytocin in breast cancer, but not benign fibrocystic breast disease. These data set the stage for neuropeptides to serve as growth regulatory factors for breast cancer. Research has indicated that vasopressin and oxytocin may have growth promoting and growth inhibiting influences on breast cancer, respectively. We have demonstrated the expression of mRNAs for the oxytocin receptor, and all known vasopressin receptor subtypes in breast cancer cells. These findings indicate that multiple receptor interactions must be taken into account when deciphering the influence of these peptides on cancer cells. In addition, we have found vasopressin-induced calcium mobilization and preliminary results suggest vasopressin is capable of inducing activation of the MAPK cascade in breast cancer. Such results implicate vasopressin VACM and/or $V_{1a/1b}$ receptors in vasopressin growth effects. With our recent cloning of the VACM receptor from cancer cells, we are now able to design experiments where the role of this receptor in cancer cell biology can be evaluated (e.g. receptor overexpression, antisense knockout). The receptors and signal transduction events for the growth regulatory effects of hormones, like vasopressin and oxytocin, remains an understudied area of breast cancer research. Understanding the interactions of hormones with cancer cell growth regulatory mechanisms is essential to identifying potential novel diagnostic and therapeutic strategies for this cancer.

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(9) APPENDICES

The following items are located in the Appendices:

1. K.A. Longo, W.G. North, J. Du, and **M.J. Fay**. Evidence for the expression of a novel vasopressin-activated calcium mobilizing receptor (HVACM) in human breast cancer and lung cancer. 1997 World Congress of Neurohypophysial Hormones (Montreal, Canada) August 8 - 12, 1997.

2. **Fay, M.**, Du,J., Longo, K., and North, W. The role of vasopressin and oxytocin hormones in breast cancer. The Department of Defense Era of Hope Breast Cancer Research Program Meeting (Washington, D.C.) October 31 - November 4, 1997.

3. A copy of our genbank submission for homo sapiens vasopressin-activated calcium mobilizing putative receptor protein (VACM-1) mRNA and protein, genbank accession number AF017061.

Abstract Form A

1997 World Congress of Neurohypophysial Hormones

Montreal, QC, Canada

August 8 - 12, 1997

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Abstract title Evidence for the expression of a novel vasopressin-activated calcium mobilizing receptor (HVACM) in human breast cancer and lung cancer cell lines

Authors including presenter (Please underline presenter's name)	Institutions
1 Kenneth A. Longo	1 Dartmouth Medical School
2 William G. North, Ph.D.	2 Dartmouth Medical school
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4 Michael J. Fay, Ph.D.	4 Dartmouth Medical School

Please submit additional authors on a separate sheet of paper

Please type text of abstract ONLY (not title) within this frame

The purpose of this study was to determine if a human homologue of the rabbit vasopressin-activated calcium mobilizing (VACM-1) receptor is expressed in human cancer cells. Vasopressin (AVP) may be involved in human breast cancer and lung cancer pathophysiology, as an autocrine/paracrine factor. AVP can act through four classes of receptors: V2, V1a, V1b, and the recently cloned VACM-1, a structurally unique member of this group that contains a single transmembrane domain. (Recently, a highly homologous cDNA, termed HVACM, was cloned from human placental mRNA.) AVP induced an increase in intracellular free calcium in the breast cancer cell lines MCF-7, T47-D, and ZR-75, and in the lung cancer cell line NCI H-146. Total RNA from these cell lines and normal human tissues (kidney and lung), was used for reverse transcription polymerase chain reaction (RT-PCR) and Northern blot analysis. RT-PCR, using two primer sets designed against the rabbit VACM-1 sequence, amplified bands of the predicted sizes of 674 bp and 193 bp in all cell lines and tissues tested. Direct sequencing of PCR products obtained from H-146 revealed a high degree of identity to the rabbit VACM-1 cDNA (90%) and the human HVACM cDNA (99.5%). Northern blot analysis revealed three distinct bands (3.5, 5 and 6.5 kilobases) in the cancer cell lines. In summary, we have demonstrated the presence of mRNA for a novel AVP receptor in human cancer cell lines and normal human tissues.

THE ROLE OF VASOPRESSIN AND OXYTOCIN HORMONES IN BREAST CANCER

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Research performed in this laboratory shows that breast cancer cells produce small peptide hormones like vasopressin and oxytocin. Other laboratories have demonstrated that vasopressin and oxytocin modulate breast cancer cell growth. How these peptides influence breast cancer cell growth and the cell surface hormone receptors involved are unknown. Using the technique of reverse transcription polymerase chain reaction (RT-PCR) we were able to demonstrate mRNA(s) for the oxytocin receptor, the vasopressin-activated calcium mobilizing receptor (VACM), the V1a vasopressin receptor, the V1b vasopressin receptor, and the V2 vasopressin receptor in a number of breast cancer cell lines. Collectively these results indicate that breast cancer cells produce neuropeptide receptors through which vasopressin and oxytocin might act to influence breast cancer cell growth. To examine cellular changes associated with receptor activation and cell growth, we evaluated neuropeptide-induced changes in intracellular free calcium and activation of the mitogen-activated protein kinase cascade. Results using two cultured breast cancer cell lines shows that vasopressin, but not oxytocin, was capable of increasing intracellular free calcium. Data using the MCF-7 breast cancer cell line suggest that one manner through which vasopressin might stimulate breast cancer cell growth is through activation of the MAP kinase cascade. Identifying hormones involved in breast cancer cell growth, the hormone receptors through which these peptides act, and the cellular changes associated with receptor activation is crucial to identifying novel strategies for the treatment of breast cancer.

Using the technique of RT-PCR evidence was obtained for the expression of mRNA(s) for a number of vasopressin and oxytocin receptor subtypes in cultured breast cancer cell lines. Using two primer pairs based on the sequence of the VACM receptor, PCR products of the predicted sizes of 674 bp and 193 bp were amplified from MCF-7, T47D, and ZR-75 breast cancer cell lines. Using a primer pair based on the oxytocin receptor, a PCR product of the predicted size of 391 bp was amplified from BT549, MCF-7, MDA-MB-231, T47D, and ZR-75 breast cancer cell lines. From the ZR-75, BT549, and MCF-7 cell lines a PCR product of the predicted size of 862 bp was amplified using primers for the V2 vasopressin receptor. In addition, using the V2 receptor primers, a PCR product which is approximately 100 bp larger than expected was amplified from these three cell lines. It is believed that this PCR product represents an incompletely spliced mRNA species containing the second intron. Using Primer pairs that amplify a 239 bp PCR product for the V1b vasopressin receptor, a product of the predicted size was amplified from the MCF7 breast cancer cell line. Preliminary PCR results using a primer pair based on the V1a vasopressin receptor indicate that a PCR product of the predicted size of 408 bp was amplified from the T47D breast cancer cell line. The identity of the VACM and V1b PCR products has been verified by direct DNA sequencing of the PCR products. Northern blot analysis for VACM using RNA from the ZR-75, MCF-7, and T47D cell lines indicates RNA species of ~ 3.5, 5, and 6.5 Kb. Using indo-1 AM loaded ZR-75 and T47D breast cancer cells neuropeptide induced changes in intracellular free calcium was monitored using flow cytometric analysis. Vasopressin (0, 10 nM, 100 nM, and 1,000 nM) was administered after approximately 20 seconds of baseline. In both cell lines vasopressin at the 100 nM and 1,000 nM doses induced a rise in intracellular-free calcium as indicated by an increase in the 405nm/485nm ratio. At all the doses studied oxytocin (10 nM, 100 nM, 1,000 nM) did not cause a noticeable rise in intracellular-free calcium in the ZR-75 and T47D cell lines. Treatment of MCF-7 breast cancer cells with 100 nM and 1,000 nM vasopressin resulted in a dose-dependent increase in tyrosine phosphorylated MAP kinase as determined by Western blot analysis.

Both in vivo and in vitro results indicate that neuropeptides like vasopressin can serve as growth modulating agents for breast cancer. Research performed in this laboratory indicates that neuropeptides, like vasopressin and oxytocin, are produced by breast cancer cells. Collectively these results suggest that neuropeptide hormones may serve as autocrine/paracrine factors for breast cancer. The results obtained in these studies provide further support for a role of vasopressin and oxytocin as paracrine/autocrine factors for breast cancer since mRNA(s) for a number of receptors for these hormones are expressed in cultured breast cancer cells. Vasopressin treatment causes a rise in intracellular free calcium in two cultured breast cancer cell lines, suggesting that the hormone might be activating VACM, V1a, or V1b receptor subtypes. Experimental results obtained with the MCF-7 breast cancer cell line suggest that the influence of vasopressin on breast cancer cell growth observed in vivo and in vitro may be due to activation of the MAP kinase cascade. These results further support a role for neuropeptide hormones like vasopressin and oxytocin in breast cancer pathophysiology. Identifying hormones involved in breast cancer cell growth, the hormone receptors through which these peptides act, and the cellular changes associated with receptor activation is crucial to identifying novel strategies for the treatment of breast cancer.

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This work was supported by the U.S. Army Medical Research and Material Command under DAMD 17-94-j-4131.

THE ROLE OF VASOPRESSIN AND OXYTOCIN HORMONES IN BREAST CANCER

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This laboratory has demonstrated that fixed breast cancer biopsy specimens exhibit positive immunoreactivity for vasopressin and oxytocin gene-related products using the technique of immunohistochemistry and antibodies directed against different regions of the vasopressin and oxytocin prohormones. In addition, both in vitro and in vivo research indicate that neuropeptides, like vasopressin and oxytocin, modulate breast cancer cell growth. Taken together these results suggest that vasopressin and oxytocin may serve as autocrine and/or paracrine growth modulators for breast cancer cells. However, the receptors and signal transduction pathways through which vasopressin and oxytocin act to influence breast cancer cell growth remain unknown. The purpose of this research is to determine if breast cancer cells express vasopressin and oxytocin receptors, and to evaluate vasopressin- and oxytocin-induced signal transduction in breast cancer cells.

To evaluate which vasopressin and oxytocin receptor subtypes are expressed by breast cancer cells the technique of reverse-transcription polymerase chain reaction (RT-PCR) was used with primer pairs specific for the oxytocin receptor, the V1a vasopressin receptor, the V1b vasopressin receptor, the V2 vasopressin receptor, and the vasopressin-activated calcium mobilizing receptor (VACM). The VACM and V1b receptor PCR products were confirmed by direct DNA sequencing. To study vasopressin and oxytocin induced changes in intracellular-free calcium, breast cancer cells were loaded with indo-1 AM, and neuropeptide-induced changes in intracellular free calcium monitored over a four minute period using a Becton Dickinson Facstar Plus flow cytometer [excitation 356 nm, emissions 405 nm (calcium bound indo), and 485 nm (free indo)]. To determine if vasopressin causes activation of the mitogen activated protein kinase cascade (MAP kinase), MCF-7 breast cancer cells were stimulated with vasopressin, and activated (phosphorylated) MAP Kinase evaluated by western blot analysis.

Keywords: Breast Cancer Cells, Vasopressin and Oxytocin, Vasopressin and Oxytocin Receptors, Signal Transduction.

This work was supported by the U.S. Army Medical Research and Material Command under DAMD 17-94-j-4131.

```
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  AUTHORS   Longo,K.A., North,W.G., Du,J. and Fay,M.J.
  TITLE     Direct Submission
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2341 gcataatttt gaatatcatg gacaatattt agaacccaaa ttttggagtg cttgggcaga
2401 aagttgtaaa gtttgtgctg gagaaaggtt tatttggact ttgattacat aaatattaat
2461 a
//
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